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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of:			
Applicants:	Javed Naim Agrewala et al.	Art Unit:	1645
Serial No.:	09/815,602	Examiner:	Rodney P. Swartz
Filing Date:	March 23, 2001		

For: PROCESS FOR THE PREPARATION OF A VACCINE FOR THE TREATMENT OF TUBERCULOSIS AND OTHER INTRACELLULAR INFECTIONS DISEASES AND THE VACCINE PRODUCED BY THE PROCESS

DECLARATION OF JAVED NAIM AGREWALA UNDER 37 C.F.R. 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Javed Naim Agrewala, declare and say as follows:

(1) I have received the following degree(s): a M.Sc. Degree in Chemistry from Agra College, Agra, India in 1982; and a Ph.D. in Biomedical Organic Chemistry in 1986 from S.N. Medical College, Agra, India.

(2) I have been working in the field of vaccine preparation for at least 14 years* and currently hold the position of Scientist E1 at The Council of Scientific and Industrial Research.

*Work Experience:

(a) Costimulatoty molecules mediated immunomodulation: An approach to skew response against vaccines towards Th1 and Th2 cells: Research experience in the field of costimulation of Th cells. Our approach was specially directed towards regulation of Th1 and Th2 cells by the distinct costimulatory molecules expressed on the surface of antigen presenting cells. We have demonstrated that distinct regulatory mechanism operates in macrophages and B cells for delivering costimulatory signals to T cells. We also observed that melatonin, a penial gland hormone regulates the expression of the costimulatory molecule B7-1 but not B7-2 on macrophages. Further, in the case of leprosy, a mycobacterial disease, we have ascertained the potential role of B7-1 and CD28 molecules in immunosuppression. Recently, we have provided a novel insight that the delivery of costimulatory signals by APC not only activate T cells but may also influence the APC itself. We have provided a mechanism whereby signaling through B7-1 and B7-2 could deliver regulatory signals in B cells.

(b) Regulation of Memory $CD4^+$ T cells against vaccines: Currently working to understand the generation, localization and persistence of T cell memory. T cell memory may have a migratory preference for different sites. The pattern of migration and the behavior of memory cells in different tissues remain unclear. It is also unknown whether memory T cells reside only in lymphoid organs or have a capability to migrate towards non-lymphoid organs. This study is undertaken to understand the population dynamics and the functional status of $CD4^+$ naive T cells, effectors, memory and naive cells in the lymphoid organs and non-lymphoid sites.

(c) Tuberculosis Research with particular emphasis for understanding immune response for developing vaccine:

(i) A role of 38kD antigen of *M. tuberculosis* in regulating the generation of $CD4^+$ Th1 and Th2 subsets.

(ii) Isolation of *M. tuberculosis* antigens which could selectively induce the generation of Th1 and Th2 cells.

(iii) Genetic and functional restriction of Promiscuous and Monogamous Th clones generated against the peptides of 16kDa antigen of *M. tuberculosis*.

(iv) Influence of HLA-DR on the phenotype of $CD4^+$ T cells specific for an epitope of the 16-kDa α-crystallin antigen of *M. tuberculosis*.

(v) Delivery of signals from T cells to infected macrophages and vice versa.

(d) Role of liposomes in delivering antigens:
(i) The effect of delivery of antigen into the MHC-class I and class II pathways of antigen presenting cell using liposomes of different physico-chemical nature and their role in the generation of Th1 and Th2 response.
(ii) The delivery of antigen into cytosol and generation of $CD8^+$ cytotoxic T cells.

(e) Immunology of hepatitis virus derived polyvalent immunogen: In this study it was observed that antigen specific early primary humoral responses modulate immunodominance of B cell epitopes.

(f) Immuno-pharmacology and Immuno-genetics: Experience in Immuno-pharmacology and Immuno-genetics of Leprosy. This work entailed the study of the evolvement of anti-leprotic drugs and their antibodies in Lepra reaction and effective chemotherapy of leprosy. Genetic study was also carried out in 500 leprosy families to evaluate an association between leprosy or type of leprosy and HLA, the Major Histocompatibility complex of man.

(3) I am a named author in approximately 35 refereed scientific journal publications, with numerous additional publications either in press or submitted for publication.

Publications of particular relevance to this Declaration include those listed below, which details some (but by no means all) of my recent refereed publications in scientific journals.

(a) My recent refereed publications in scientific journals include the following:

1. **Agrewala, J.N.**; Suvas, S.; Singh, V.; Vohra, H.; Delivery of antigen in allogeneic cells preferentially generates $CD4^+$ Th1 cells, Clin Exp Immunol., 134 (2003)13-22.

2. Suvas, S.; Singh, V.; Sahdev, S.; Vohra, H.; **Agrewala, J.N.**; Distinct Role of CD80 and CD86 in the regulation of the activation of B cell and B cell Lymphoma, J Biol Chem., 277 (2002) 7760-75.

3. Swain, S.L.; **Agrewala, J.N.**; Brown, D.M.; Roman, E.; Regulation of memory CD4 T cells: generation, localization and persistence, Adv Exp Med Biol, 512 (2002) 113-20.

4. Raghavendra, V.; Singh, V.; Kulkarni, S.K.; **Agrewala, J.N.**; Melatonin enhances Th2 mediated immune responses: lack of sensitivity to reversal by naltrexone or benzodiazepine receptor antagonists, Mol. Cell. Biochem., 221 (2001) 57-62.

5. Raghavendra, V.; Singh, V.; Shaji, A.V.; Vohra, H.; Kulkarni, S.K. and **Agrewala, J.N.**; Melatonin provides signal 3 to unprimed CD4$^+$ T cells but failed to stimulate LPS primed B cell, Clin. Exp. Immunol., 124 (2001) 414-22.

6. Owais, M.; Masood, A.K.; **Agrewala, J.N.**; Bisht, D. and Gupta, C.M.; Use of liposomes as an immunopotentiating delivery system: in perspective of vaccine development, Scand. J. Immunol., 54 (2001) 125-32.

7. Raghava, G.P.S. and **Agrewala, J.N.**; A web-based method for computing endpoint titter and concentration of antibody/antigen; Biotech Software Internet Report, 2 (2001)196-7.

8. Raghavendra, V.; **Agrewala, J.N.** and Kulkarni, S.K.; Melatonin reversal of lipolysaccharides-induced thermal and behavioral hyperalgesia in mice, Eur. J. Pharmacol., 395 (2000) 15-21.

9. Raghavendra, V.; **Agrewala, J.N.** and Kulkarni, S.K.; Role of centrally administered melatonin and inhibitors of COX and NOS in LPS-induced hyperthermia and adipsia, Prost. Leuko. Essen. Fatty Acids., 60 (1999) 249-53.

10. **Agrewala, J.N.** and Wilkinson, R.J.; Influence of HLA-DR on the phenotype of CD4$^+$ T lymphocytes specific for an epitope of the 16-kD a-crystalline antigen of Mycobactenum tuberculosis, Eur. J. Immunol., 29 (1999) 1753-61.

11. Das, G.; Vohra, H.; Saha, B.; **Agrewala, J.N.** and Mishra, G.C.; Apoptosis of Th1-like cells in experimental tuberculosis, Clin. Exp. Immunol., 115 (1999) 324-8.

12. Das, G.; Vohra, H.; Saha, B.; **Agrewala, J.N.** and Mishra, G.C.; Leishmania donovani infection of a susceptible host results in apoptosis of Th1-like cells: rescue of anti-leishmanial CMI by providing Th1-specific bystander costimulation, Microbial. Immunol., 42 (1998) 795-801.

13. **Agrewala, J.N.** and Wilkinson, R.J.; Differential regulation of Th1 and Th2 cells by p91-110 and p21-40 peptides of the 16-kD a-crystalline antigen of Mycobacterium tuberculosis, Clin. Exp. Immunol., 114 (1998) 392-7.

14. **Agrewala, J.N.**; Upma and Banyal, H.S.; A 24,000g sedimant of Plasmodium berghei induces IL-1 response in mice and exhibits protection against malaria infection, Parasitology (Hung)., 31 (1998) 13-8.

15. **Agrewala, J.N.**; Suvas, S.; Joshi, A.; Bhatanagar, A.; Vinay, D.S. and Mishra, G.C.; M150 modulates the costimulatory signals delivered by B cells to T cells and enhances their ability to help B cells, J. IFN. Cyto. Res., 18 (1998) 297-304.

16. **Agrewala, J.N.**; Suvas, S.; Verma, R.K. and Mishra, G.C.; Differential effect of anti-B7-1 and anti-B7-2 antibodies in restricting the delivery of costimulatory signals from B cells and macrophages, J. Immunol., 160 (1998) 1067-77.

17. Shaji, A.V.; Kulkarni, S.K. and **Agrewala, J.N.**; Regulation of secretion of IL-4 and IgG1-isotype by melatonin stimulated ovalbumin specific T cells, Clin. Exp. Immunol., 19 (1998) 181-5.

18. **Agrewala, J.N.**; Kumar, B. and Vohra, H.; Potential role of B7-1 and CD28 molecules in immunosuppresion in leprosy, Clin. Exp. Immunol., 111 (1998) 56-63.

19. **Agrewala, J.N.**; Deacock, S.; Jurcevic, S. and Wilkinson, R.; Peptide recognition by T cell clones of HLA-DRB1*1501/*901 heterozygous donor is promiscuous only between parental alleles, Hum. Immunol., 55 (1997) 34-8.

20. Pitchappan, R.M.; **Agrewala, J.N.**; Dheenadayalan, V. and Ivanyi, J.; MHC-restriction in Tuberculosis, J. Biosci., 22 (1997) 47-56.

21. Ghei, S.K.; Sengupta, U.; **Agrewala, J.N.**; Kailash, S.; Gunasekharan, N.; Sudhakar, K. S.; Desikan, K.V.; Shepard, C.C. and Shinnick, T.; 1996 Association of HLA antigens with leprosy, pp. 273-278. In Singh, J. (eds), Current Concepts in Human Genetics, Guru Nanak Dev University, Amritsar.

22. Jurcevic, S.; Travers, P.; Hills, A.; **Agrewala, J.N.**; Moreno, C. and Ivanyi, J.; Distinct conformations of a peptide bound to HLA-DR1 or DRB5*0101 suggested by molecular modelling, Int. Immunol., 8 (1996) 1807-14.

23. **Agrewala, J.N.**; Owais, M.; Gupta, C.M. and Mishra, G.C.; Antigen incorporation into liposomes results in the enhancement of IL-4 and IgG1 secretion: evidence for preferential expansion of Th-2 cells, Cytokines Mol. Ther., 2 (1996) 59-65.

24. **Agrewala, J.N.** and Mishra, G.C.; A 38kDa antigen of Mycobacterium tuberculosis predominantly induces the secretion of interleukin-2, interferon-gamma and IgG2a antibodies, Microbiol. Immunol., 39 (1995) 801-8.

25. Raghava and **Agrewala, J.N.**; Method for determing the affinity of monoclonal antibody using non-competitive ELISA: a computer program, J. Immunoassays., 15 (1994) 115-28.

26. Vijayakrishnan, L.; Kumar, V.; **Agrewala, J.N.**; Mishra, G.C. and Rao, K.V.S.; Antigenspecific early primary humoral responses modulate Immunodominance of B cell epitopes, J. Immunol., 153 (1994) 1613-25.

27. **Agrewala, J.N.**; Vinay, D.S.; Joshi, A. and Mishra, G.C.; A 150-kDa molecule of murine macrophage membrane stimulates interleukin-2 and interferon-g production and proliferation of ovalbumin-specific $CD4^+$ T cells, Eur. J. Immunol., 24 (1994) 2092-7.

28. Ghei; **Agrewala, J.N.**; Sengupta, U. and Sudhakar, K.S.; Group specific component in Erythema Nodosum Leprosum, Ind. J. Lepr., 65 (1993) 323-5.

29. **Agrewala, J.N.**; Raghava, G.P.S. and Mishra, G.C.; Measurement and computation of murine interleukin-4 and interferon-g by exploiting the unique abilities of these lymphokines to induce the secretion of IgG1 and IgG2a, J. Immunoassays., 14 (1993) 83-97.

30. Raghava, Joshi, A. and **Agrewala, J.N.**; Calculation of antibody and antigen concentrations from ELISA data using a graphical method, J. Immunol. Methods., 153 (1992) 263-4.

31. **Agrewala, J.N.**; Sinha, S. and Sengupta, U.; Caution when standardizing serum antibody competition assays, Trans. Roy. Soc. Trop. Med. Hyg., 84 (1990) 137-8.

32. **Agrewala, J.N.**; Sinha, S.; Ghei, S.K.; Katoch, K.; Girdhar, B.K. and Sengupta. U.; Demonstration of anti-dapsone antibody in leprosy patients, Int. J. Lepr., 57 (1989) 687-90.

33. **Agrewala, J.N.**; Ghei, S.K.; Sudhakar, K.S.; Girdhar, B.K. and Sengupta, U.; Human Leukocyte antigen and Erythema Nodosum Leprosum, Tissue Antigens., 33 (1988) 486-7.

(4) I am one of the named co-inventors of the presently pending patent application (USSN 09/815,602).

(5) I have been asked to comment on the contents of the application and particularly on the breadth of the disclosure made in the presently pending patent application and on what a person skilled in the art would have understood by that disclosure on March 23, 2001 (the filing date of the present application) and, whether such a person skilled in the art would have had a reasonable expectation of being able to make vaccines which would be effective against a variety of diseases.

(6) Based on the disclosure contained in the present application, I conducted the experiments detailed below and obtained the results as discussed below:

Data showing protective efficacy of the vaccine

(a) Figure 1: Predominance of TH1-Like responses after administration of atlogeneic (AMTV), syngeneic (SMTV) and xenogeneic (XMTV) vaccines

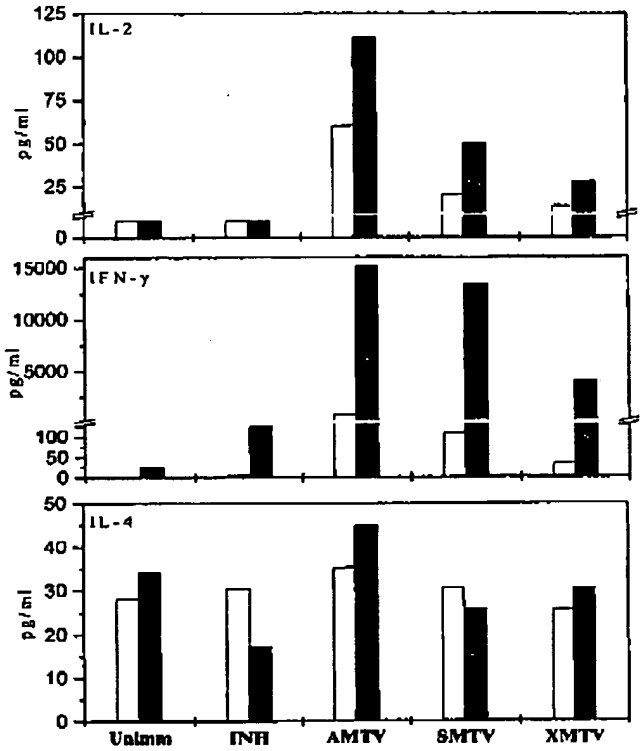

Fig 1: *Predominance of Th1-like responses after administration of allogeneic (AMTV), syngeneic (SMTV) and xenogeneic (XMTV) vaccines.* The secretion of IL-2, IFN-γ and IL-4 in response to *in vitro* stimulation with PPD at 10 μg/ml (gray bars) or medium alone (open bars) are shown from the splenocytes pooled from mice injected with different preparations of vaccines. The assay was set seven days after the final syngeneic booster. INH indicates mice immunized with vaccine prepared by treating *M. tuberculosis* by isoniazid and "unimm" indicates control group of unvaccinated mice. Cytokines ELISA was done on supernatants collected from different experimental and control cultures and pooled from the triplicate wells. The results shown are the representative of 3-4 experiments.

in syngeneic (J774), allogeneic (BMC-2) and xenogeneic (THP-1) macrophages and treating the preparation with amikacin, isoniazid and gamma irradiation. A secondary booster was given with a vaccine prepared using syngeneic macrophages. The animals were challenged with *M. tuberculosis* 7 days after the secondary booster and sacrificed 30 days post-challenge. The splenocytes were isolated and cultured *in vitro* with PPD and supernatants were collected after 48 hours from the experimental and control cultures, pooled from the triplicate wells and

were used to measure IL-2, IFN-γ and IL-4 by ELISA. The gray/blackish bars in Figure 1 indicate cells cultured with PPD and open bars represent cells cultured with medium alone.

Based on the results detailed in Figure 1, immunization with *M. tuberculosis* infected macrophage cell lines showed predominant increases in the secretion of IFN-γ and IL-2. Studies by several groups have shown that development of effective immunity to intracellular pathogens like *M. tuberculosis* is strongly associated with marked expansion of Thl lymphocytes as evidenced by increased production of IFN-γ and IL-2.

The results show that the splenocytes from different vaccinated groups (AMTV, SMTV and XMTV) on *in vitro* stimulation with PPD induced very high levels of IFN-γ and IL-2 secretion as compared to the splenocytes from unimmunized animals or animals immunized with isoniazid treated mycobacterium (INH). No significant change was observed in IL-4 secretion in any of the vaccinated groups (Figure 1).

Thus, this study shows that our vaccine strategy specifically induces Th1-type of immune response. Th1 cells are known to induce favorable response in protection against *M. tuberculosis*.

(b) Figure 2: Immunization with mycobacterium entrapped in macropkages confer protection to Balb/c mice on subsequent infection with *M. tuberculosis*

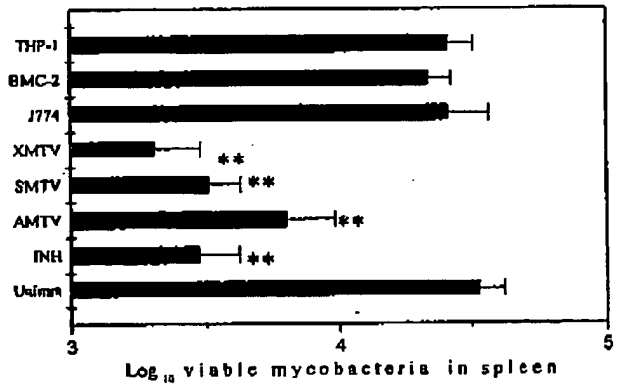

Fig 2: *Immunization with mycobacteria entrapped in macrophages confers protection to Balb/c mice on subsequent infection with H37Rv.* The animals were challenged with mycobacterium 7 days after the final syngeneic booster. Animals were sacrificed 30 days post challenge and dilution plating of the spleen was done. The control groups were immunized with uninfected cells (THP-1, BMC-2 and J774). The data represents mean $\log_{10}$ viable cfu (±SEM) obtained from two experiments with five mice per group. *** $P < 0.001$ as compared to unimmunized group.

Animals were immunized with vaccine prepared by culturing *M. tuberculosis* in syngeneic (J774), allogeneic (BMC-2) and xenogeneic (THP-1) macrophages and treating the preparation with amikacin, isoniazid and gamma irradiation. A secondary booster was given with the vaccine prepared by culturing syngeneic macrophages. The animals were challenged with mycobacterium 7 days after the secondary booster and sacrificed 30 days post-challenge and dilution plating of the spleen homogenate was done to enumerate the viable bacteria by cfu. The control groups were immunized with uninfected cells (THP-1, BMC-2 and J774). The data represent mean $\log_{10}$ viable cfu (±SEM) obtained from experiments with five mice per group. *** represents $P < 0.001$ as compared to an un-immunized group.

Based on the results detailed in Figure 2, vaccinated mice show significant decrease in the load of mycobacterium upon subsequent infection with *M. tuberculosis.*

This experiment was performed to ascertain whether *M. tuberculosis* cultured in syngeneic, allogeneic and xenogeneic macrophages being used as vaccine, could render protection against infection with *M. tuberculosis.* Thus, to study the

protective efficacy of different vaccines used in our study, the animals were immunized with different preparation of vaccines (AMTV, SMTV and XMTV). Seven days after the secondary syngeneic booster, mice were challenged intra-peritoneally with *M. tuberculosis*. The peak of infection was established after 30 days of intra-peritoneal challenge. On day 30, the mice were sacrificed and viability of the bacteria in the spleen was enumerated by colony forming units (cfu). The number of mycobacteria recovered from the spleens of mice immunized with different preparation of vaccines was substantially reduced as compared to unvaccinated controls. The groups immunized with uninfected cells only (J774, BMC-2 and THP-1) failed to show any significant change in the bacterial load as compared to un-immunized mice. All the vaccines used in the study showed highly significant ($p<0.001$) decrease in the bacterial load in the mice infected with live M. *tuberculosis*.

(c) Figure 3: Vaccinated Balb/c mice survive upon infection with *S. typhimurium*

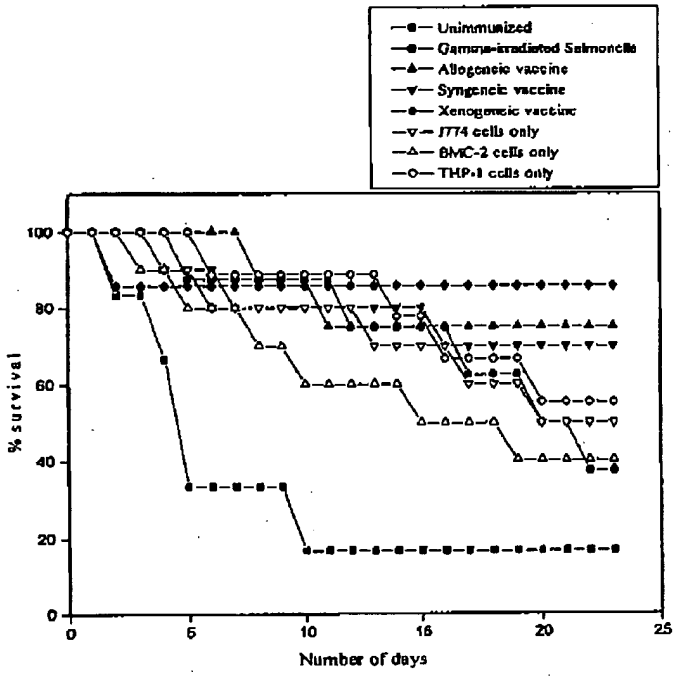

Fig 3: *Vaccinated Balb/c mice survive S. typhimurium infection*- Different groups of Balb/c mice were immunized with gamma-irradiated *Salmonella*, allogeneic, syngeneic or xenogeneic vaccines and their respective uninfected cell lines as controls. The mice were challenged with 2.5 x 10^5 cfu of *Salmonella typhimurium* intraperitoneally. Each group comprised of 8-10 mice. The results are representative of four independent experiments.

Different groups of Balb/c mice were immunized either with allogeneic or syngeneic or xenogeneic vaccines or gamma-irradiated *S. typhimurium* and their respective uninfected cell lines as controls. The mice were challenged with 2.5 x 10^5 cfu of *S. typhimurium* intra-peritoneally and protective efficacy of the vaccine was monitored by examining the survival of the mice. Each group comprised 8 to 10 mice.

Based on the results detailed in Figure 3, immunization with a vaccine prepared in accordance with the claimed invention also protects experimental animals from another intra-cellular pathogen S. *typhimurium*.

This experiment was performed to ascertain whether vaccine prepared using *S. typhimurium* infected and irradiated syngeneic, allogeneic and xenogeneic macrophages could render protection against subsequent infection.

Different groups of Balb/c mice (IA^d haplotype) were immunized with different preparations of vaccine viz. allogeneic vaccine i.e. salmonella infected BMC-2 ($H\text{-}2^b$) cells, syngeneic vaccine i.e. salmonella infected J774 (IA^d) cells and xenogeneic vaccine, i.e. salmonella infected THP-1 cells (see Figure 3). In the control groups of mice, Balb/c were injected with uninfected cells (J774, BMC-2 and THP-1). Seven days post-immunizations of final syngeneic booster, the animals were challenged with a lethal dose (2.5 x 10^5 cfu/animal) of *S. typhimurium*. The percentages survival was then recorded for 23 days post-challenge (Figure 3). Animals vaccinated with xenogeneic vaccine showed maximum survival (85.7%), followed by allogeneic (75%), and syngeneic (70%) vaccinated groups. The groups immunized with uninfected cells only or gamma-irradiated salmonella did not show significant protection. In an un-immunized group, 83.3% animals were dead by day 10 after

the infection.

(d) Figure 4: Vaccinated FVB mice survive upon infection with *S. typhimurium*

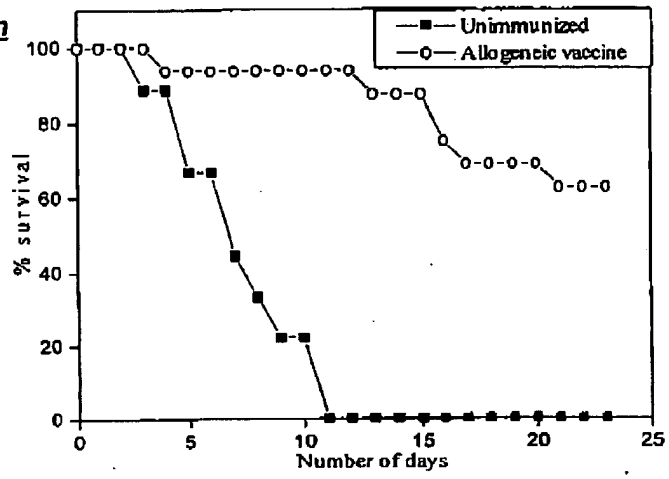

Fig 4: *FVB mice immunized with allogeneic vaccine show protection on challenge with S. typhimurium.* Survival of FVB mice immunized with allogeneic vaccine (8-10 animals per group), challenged with 2.5 x 10^5 cfu of *Salmonella typhimurium* intraperitoneally. The results are representative of three independent experiments.

Similarly FVB mice were immunized with allogeneic vaccine (8 to 10 animals per group) and challenged intra-peritoneally with 2.5 x 10^5 cm of *S. typhimurium* and protective efficacy of the vaccine was monitored by examining the survival of the mice.

As discussed above, a vaccine made in accordance with the presently claimed invention was also tested in FVB mice (IA^q haplotype), which are genetically dissimilar from Balb/c mice (IA^d haplotype). FVB mice were immunized with allogeneic vaccine, i.e. J774 (LA) cells infected with *S. typhimurium.* Seven days after the second booster, the animals were infected with a lethal dose (2.5 x 10^5 cfu/animal) of *S. typhimurium.* The percentage survival of infected mice was then recorded for 23 days post-challenges (Figure 4). A control group of unimmunized animals was also included in the study. All the un-immunized FVB mice were dead by day 11 after the infection, where as the vaccinated group showed 62.5% survival even after 23 days of infection.

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I, Javed Naim Agrewala, declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated Oct 6, 2003

Javed Naim Agrewala